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DETERMINATION OF SPECTINOMYCIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic method for the quantitative determination of spectinomycin in the presence of actinospectinoic acid and actinamine is described. A combination of paired ion chromatography, post column oxidation, post column derivatization and fluorometric detection is employed. Total assay time is less than 15 min. The assay was designed for determining spectinomycin in samples of spectinomycin finished products, process samples and fermentation beers. Quantitative results from this procedure are compared to those obtained by a turbidimetric microbiological assay and are in good agreement.

INTRODUCTION

Spectinomycin is a broad-spectrum aminocyclitol antibiotic isolated from fermentation broth of *Streptomyces spectabilis*[1–4]. This antibiotic is polyfunctional and is stable only within a narrow pH range. Spectinomycin and most of its degradation products are water-soluble compounds which lack UV-absorbing chromophores. Preparation of homogenous UV-absorbing derivatives of these substances in a quantitative manner has been reported to be a very arduous task[5]. As a result, a rapid and reliable, chromatographic method of analysis has not been heretofore developed.

Two chromatographic methods of analyses for determining the purity of modified spectinomycins and degradation products have been employed in this laboratory[5,6], These methods were high-performance liquid chromatography (HPLC) and gas chromatography (GC). In the former, the compounds were resolved on ion-exchange resin and detected by a differential refractometer or a photoelectric polarimeter. In the latter the compounds were derivatized with hexamethyldisilazane, chromatographed on an SE-30 stationary phase and detected by a flame ionization detector. Since these procedures lacked speed, sensitivity and precision, an alternative chromatographic approach for the quantitative determination of spectinomycin and its degradation products was investigated.

Recently, HPLC methods using fluorometric detection for gentamicin[7,8] and kanamycin[9] have come to our attention and have led us to investigate the use of a simi-

lar procedure for spectinomycin. This paper describes the paired ion HPLC method with fluorometric detection for the determination of spectinomycin in the presence of actinospectinoic acid and actinamine.

EXPERIMENTAL

Equipment

A schematic diagram of the chromatographic apparatus is shown in Fig. 1. A Spectra-Physics Model 740B Pump was used to deliver the mobile phase. The assay was run on a Merck LiChrosorb RP-8 Hibar® II column (25 cm × 4.6 mm I.D.) with a precolumn (4.3 cm × 4.2 mm I.D.) packed with Merck Perisorb® RP-8 packing. Samples were injected using a Valco CV-6UHPa-N60 injection valve with a 15-μl injection loop. Two Milton-Roy Model 196-89 Minipumps equipped with Glenco pulse dampeners were used to deliver the oxidizing reagent and the fluorogenic reagent. Precolumns packed with Applied Sciences glass beads (170–230 mesh) were installed in the reagent lines to generate back pressure for the pulse dampeners. The oxidant was mixed with the column effluent in a mixing tee (Cheminert CJ 3031 fitting). This stream then passed through a thermostated reaction coil (2.0 m × 0.5 mm I.D. PTFE tubing) at 100°. The oxidized column effluent was mixed with the

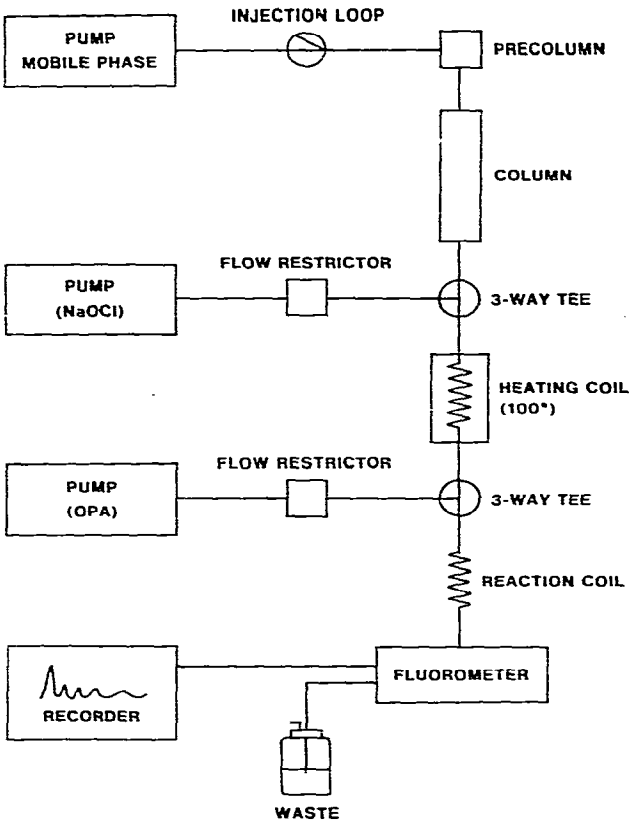


Fig. 1. Schematic diagram of the HPLC apparatus used for spectinomycin determinations.

fluorogenic reagent in a second mixing tee (Cheminert fitting CJ 3031) and then was passed through a second reaction coil (2.0 m × 0.5 mm I.D. PTFE tubing) at ambient temperature. The effluent then was passed into the Perkin-Elmer 204A fluorometer. Excitation wavelength was 350 nm and emission wavelength was 450 nm. The detector signal was processed using the Hewlett-Packard 3354A data system and a Hewlett-Packard 7153A recorder.

Reagents

Spectinomycin hydrochloride*, actinamine and actinospectinoic acid were obtained from The Upjohn Company (Kalamazoo, Mich., U.S.A.), *o*-phthalaldehyde (Fluoropa, manufactured by Durrum) from Pierce (Rockford, Ill., U.S.A.), 2-mercaptoethanol from Sigma (St. Louis, Mo., U.S.A.), sodium heptanesulfonate from Regis (Morton Grove, Ill., U.S.A.), and sodium hypochlorite (Clorox®, manufactured by The Clorox Company, Oakland, Calif., U.S.A.) from a local vendor. Water was double distilled. All other chemicals were of reagent grade. *o*-Phthalaldehyde reagent solution (OPA) was prepared by the method of Benson and Hare[10]. The oxidizing reagent contained 0.01 *M* sodium hypochlorite in 0.4 *M* potassium borate buffer, pH 10.4. Solutions of antibiotic and by-products were freshly prepared in distilled water at concentrations of 50–900 μg/ml.

Mobile phase

The mobile phase contained 0.02 *M* sodium heptanesulfonate, 0.2 *M* sodium sulfate and 0.1 % acetic acid in water. The mobile phase, after it has been filtered and degassed, was pumped through the column at a flow-rate of 2 ml/min. The oxidizing reagent and the fluorogenic reagent each are pumped into the mixing tees at a flow-rate of 0.5 ml/min.

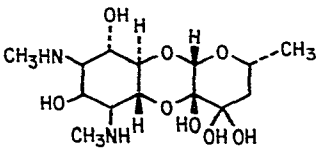
RESULTS AND DISCUSSION

Unlike other aminoglycoside antibiotics for which fluorometric detection has been used[7–9], spectinomycin contains no primary amino groups[10]. There are two secondary amino groups in the actinamine portion of this molecule (see Fig. 2)[11]. Therefore, this antibiotic does not react directly with *o*-phthalaldehyde to form a fluorescent species.

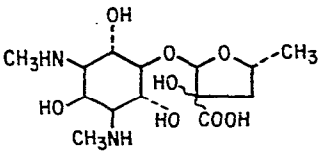
In order to detect spectinomycin via fluorescence, we considered two alternatives. The first possibility was to find a fluorogenic reagent that would react with secondary amines in aqueous solution. Dansyl chloride is the most commonly used fluorogenic reagent for reaction with secondary amines; however, it and its by-products are fluorescent. The second possibility was to convert the secondary amine to a primary amine and then react it with *o*-phthalaldehyde. We chose to pursue the latter course.

Several reports in the literature described the transformation of secondary amino acids (*e.g.*, proline, hydroxyproline) to primary amines and their subsequent reaction with fluorogenic reagents[12–14]. These secondary amino acids, when treated with either chloramine-T, N-chlorosuccinimide, sodium hypochlorite or other sources

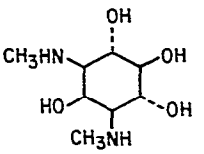
* The registered U.S. trademark of The Upjohn Company for spectinomycin, formerly known as actinospectacin, is Trobicin. Additional trademarks include Togamycin and Stanilo.



SPECTINOMYCIN



ACTINOSPECTINOIC ACID



ACTINAMINE

Fig. 2. Structures of spectinomycin and two of its degradation products.

of active halogen, underwent oxidative decarboxylation. The resulting imines were hydrolyzed to primary amines which were subsequently treated with fluorogenic reagents to form fluorescent compounds.

By analogy, spectinomycin was allowed to react with sodium hypochlorite. The degradative product was then treated with *o*-phthalaldehyde. The reaction mixture showed fluorescence with a response proportional to the spectinomycin content.

Once the problem of detecting the antibiotic was solved, the use of several different alkanesulfonate counter ions (C_5–C_7) in the mobile phase was investigated. It was concluded that sodium heptanesulfonate yielded the best separation of spectinomycin from its by-products. Typical chromatograms of a sample of spectinomycin and of spectinomycin fermentation beer are shown in Fig. 3 and 4.

It should be noted that there is a small peak in the chromatogram in Fig. 3 labeled "unknown". When a freshly prepared solution of the antibiotic is chromatographed immediately, the "unknown" peak is barely detectable. After 30 min this peak reaches a steady state which amounts to *ca.* 8% of the spectinomycin peak. This unknown component might be one of the spectinomycin diastereomers postulated by Foley and Weigele[15,16].

Calibration of assay

Several water-soluble secondary amino compounds were investigated for use as internal standards; however, no suitable substance was found. The assay was quantitated as an external standard method. Calibration curves were constructed for spectinomycin, actinospectinoic acid and actinamine. These curves were found to be linear in the region investigated from 0.05 mg/ml to 0.9 mg/ml. The precision of the assay expressed as relative standard deviation for repeated injections of standard samples was found to be 2.2% for spectinomycin, 1.7% for actinamine and 1.2% for actinospectinoic acid.

Ten crystalline samples of spectinomycin hydrochloride were analyzed using this HPLC procedure. These sample results were compared to those obtained using a

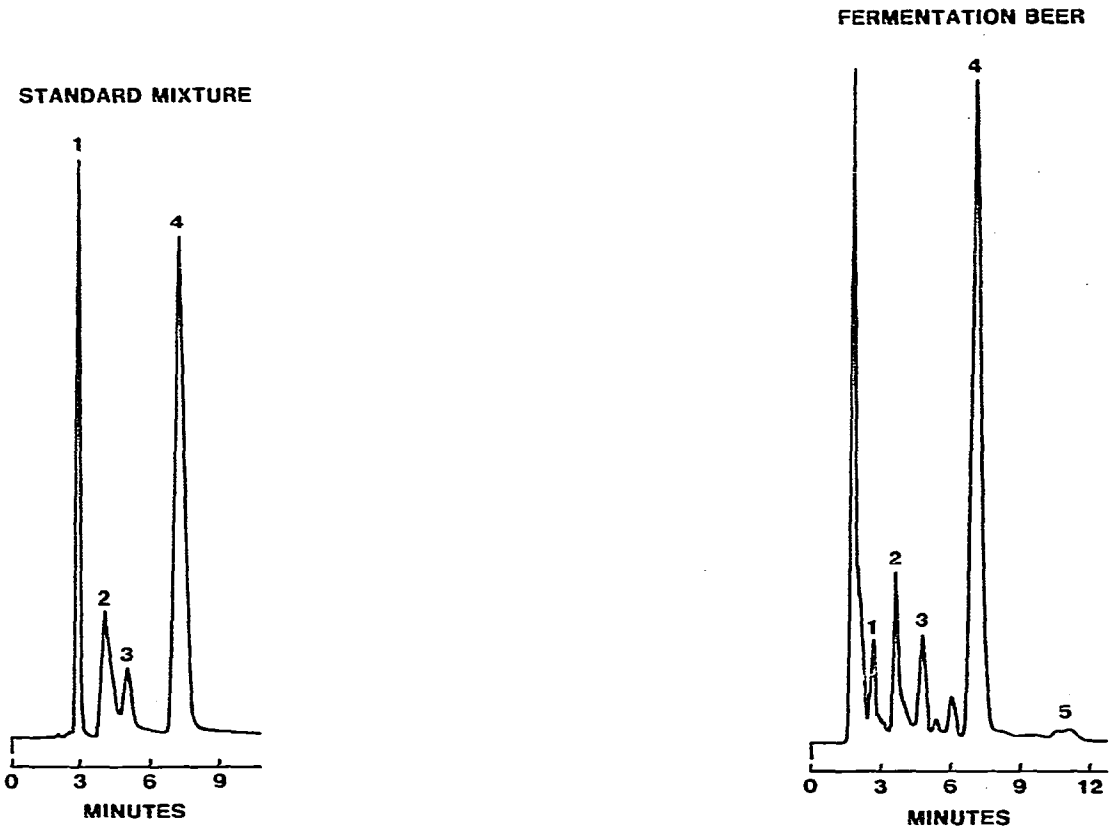


Fig. 3. Typical chromatogram of a standard mixture of spectinomycin (4), actinospectinoic acid (2), actinamine (1) and unknown (3).

Fig. 4. Typical chromatogram of spectinomycin fermentation beer. Peaks as in Fig. 3 except for dihydrospectinomycin (5).

TABLE I

COMPARISON OF THE DETERMINATION OF SPECTINOMYCIN BY HPLC AND MICROBIOASSAY

Sample	*Autoturbidimetric microbioassay (μg/mg)*	*HPLC (μg/mg)*
1	681	675
2	670	648
3	636	640
4	587	596
5	595	612
6	599	582
7	558	599
8	615	637
9	598	598
10	594	626
	Mean 613	621

turbidimetric microbiological assay. These data are summarized in Table I. The results from both assay methods agree well. The HPLC assay has advantages of speed, accuracy and sensitivity.

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